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Synthesis of 1-phenyl-3-(4'-nitrophenyl)-5-(3',4'-dimethoxy-6'-nitrophenyl)-2-Pyrazoline and Its Antibacterial Activity

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Abstract. Synthesis of pyrazoline substituted with nitro groups as antibacterial agent has been carried out by cycloaddition reaction. The compound was synthesized from chalcone and phenylhyrazine by refluxing them in 2-butanol for 24 h. The product was purified and characterized using FTIR and ^{1}H-NMR spectrometers. The result showed that pyrazoline has been succesfully synthesized in 33.06% yield. The compund has antibacterial activity againts *Bacillus subtilis* and *Shigella flexneri.* However, it has tendency of activity for Gram-negative bacteria. In conclusion, the nitro groups that substituted in aromatic ring were predicted as a part of pharmacophore.

INTRODUCTION

Electron-rich nitrogen containing hetero-cycles play an important role in diverse biological activities [1]. Pyrazole and pyrazoline are important nitrogen containing 5-membered heterocyclic compound. Pyrazoline which is well known as pyrazole derivative or 4,5-dihydropyrazole is reported possess a broad spectrum of biological activities such as anticonvulsant [2], antimicrobial [1,3,8], antiamoebic [3], analgesic [1], anti-inflammmatory [1], antidepressant [4], anxiogenic [4], inhibitors of monoamine oxidase [4], fungisical [5], insecticidal [5], antitumor [6], and antinociceptive [7]. Therefore, the prevalence of pyrazole core in biologically active molecules has stimulated the need for elegant and efficient ways to make these heterocyclic lead. Surprisingly, pyrazoline exhibited various biological activities, i.e. analgesic, anti-inflammatory and antimicrobial since the compound possessing all three activities is not common [1]. In addition, microbial infections often produce pain and inflammation, hence the research that involved synthesis for antibacterial agent from pyrazoline scaffold must be conducted.

Resistance of microorganism (bacteria, fungus, virus or parasite) was indicated by no longer responses of medical standard treatment [9]. This means that the infections cannot be handled or controlled. Besides that, the pain may spread and the risk that caused by the infections increased. In addition, the possibility of death increased twice greater than the infection of non-resistant bacteria. Very high rates of resistance have been observed in all WHO regions in common bacteria (for example, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*) that cause common health-care associated and community-acquired infections (urinary tract infections, wound infections, bloodstream infections and pneumonia [9]. The resistances of microbial, especially bacterial against β-lactams antibacterial have reported few decades ago [10,11]. Generally, there are mechanisms of bacterial became resistant, such as acquisition of exogenous resistance genes [11], mutations in side of drug target, over-production of drug targets, modification of cell permeability by loss or mutagenesis of porins, and drug inactivation by the enzyme degradation [12]. From the fact above, the design and synthesis of new antibacterial are urgently needed.

Substituents of p-nitrophenyl and p-bromophenyl at C_3 position in pyrazoline ring increased antibacterial activity [13]. Tanitame et al. [10] reported that the increased in lipofilicity of pyrazoline compound had role in antibacterial activity. Besides that, methoxyphenyl substituted at C5 could increase the strength of antibacterial [14]. This study focused on synthesis, characterization, and antibacterial test of pyrazoline derivative that was generated from cyclization of chalcone with

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hydrazine. Chalcone was synthesized in previous research [15] from p-nitroacetophenone and veratraldehyde which has been nitrated. Synthesis of pyrazoline can be performed by Michael addition or cycloaddition reaction in acid/alkaline medium or only in alchoholic solvents. Baluja and Chanda [13] had conducted synthesis of novel pyrazoline derivatives from the reaction of α-β unsaturated ketone with hydrazine monohydrate ($NH_2NH_2 \cdot H_2O$) in ethanol, yielded 4,5-dihydro-1H-pyrazol-5-yl-6-flouroquinoline in 59-76%. Synthesis of thioacetylpyrazolines have been produced by reaction of trienylchalcones with hydrazine hydrate (1: 2) in ethanol and refluxed for 3 hours to get the products in 48-71% [16]. Synthesis of pyrazoline in presense of glacial acetic acid had been conducted by Dipankar [17], to get product in 46-67% yield. Besides that, for the less reactive reactants, the reaction can be accelerated by the addition of sulfuric acid as catalyst [14].

EXPERIMENTAL

Materials

1-(4'-nitrophenyl)-3-(3',4'-dimethoxy-6'-nitrophenyl)-2-propen-1-one namely chalcone **1**, 2-butanol, phenylhydrazine, n-hexane, and dichloromethane (DCM). All materials were used obtained from E. Merck with pro analysis quality (pa). Antibacterial test materials: technical agar, nutrient broth (NB), medium nutrient agar (NA), tetracycline, alcohol 70%, and dimethylsulfoxide (DMSO).

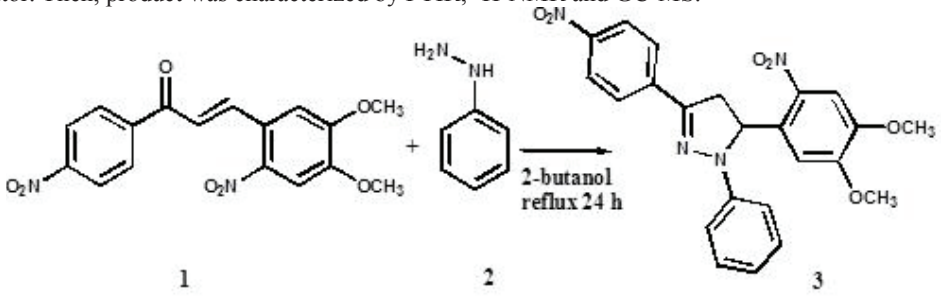
Instrumentations

Melting points are uncorrected (Electrothermal 9100), infrared spectrometer (FTIR Shimadzu Prestige 21), proton nuclear magnetic resonance spectrometer (NMR, JEOL, JNMECA ^{1}H 500 MHz), internal standard TMS.

Experimental Procedure

The chalcone **1** was prepared by reacting 4-nitroacetophenone with 6-nitroveratraldehyde in the presence of a base (NaOH) by Claisen Schmidt condensation15.

Synthesis of pyrazoline **3** was conducted by reacting 0.18 g (0.5 mmol) of chalcone **1**, 0.16 g (1.5 mmol) phenylhydrazine in 10 mL 2-butanol and refluxing for 24 hours (shown in Scheme 1). Purification was done by column chromatography using n-hexane:DCM (1: 1) as eluent. The solid that formed was filtered with Buchner filter, washed with cold distilled water and dried in desiccator. Then, product was characterized by FTIR, ^{1}H-NMR and GC-MS.



SCHEME 1. Reaction of synthesis pyrazoline **3**

1-phenyl-3-(4'-nitrophenyl)-5-(3',4'-dimethoxy-6'-nitrophenyl)-2-pyrazoline (3). Red solid. Yield 33.06%. FTIR (cm^{-1}, KBr): 2924, 2854 (C-H pyrazoline ring), 1597 (C=N pyrazoline ring), 1512, 1334 (NO_2), 1273, 1064 (C-O ether), 1072 (C-N pyrazoline ring). ^{1}H-NMR (500 MHz, $CDCl_3$) δ (ppm): 3.17 (dd, 1H, J = 6.50, 17.50 Hz, H_a pyrazoline ring), 3.70 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 4.19 (dd, 1H, J = 12.30, 17.50 Hz, H_b pyrazoline ring), 6.11 (dd, 1H, J = 6.50, 12.50 Hz, H_x pyrazoline ring), 6.76 (s, 1H, Ar-H), 6.89 (t, 1H, J = 7.65 Hz, Ar-H), 7.00 (d, 2H, J = 7.75 Hz, Ar-H), 7.22 (t, 2H, Ar-H), 7.79 (s, 2H, Ar-H), 7.86 (d, 2H, J = 9.10 Hz, Ar-H), 8.25 (d, 2H, J = 8.45 Hz, Ar-H). GC-MS (purity 74.06%). Ms relative intensity (m/z): 416 (base peak, nitro groups were reduced into nitroso group), 401, 386, 369, 341, 323, 221, 155, 128, 77, 51.

Antibacterial Test

Nutrient agar medium was used as the culture medium. Sterilization of medium was done by autoclaving at 15 psi pressure and the temperature was maintained at 121 °C for 15 minutes. Antibacterial test was performed by agar well-diffusion against Gram positive (*Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*) and negative (*Escherichia coli*, *Shigella flexneri*)

bacteries, tetracycline (100 ppm) as positive control and dimethyl sulfoxide (DMSO 99.9%) as negative control. The solutions of synthesized compound were prepared at the concentration of 100, 300, 500 and 1000 ppm in DMSO.

RESULTS AND DISCUSSION

Synthesis of Pyrazoline

Pyrazoline has been synthesized with the reaction that was shown in Scheme 1. Product of synthesis was red solid, presented in Fig. 1. ^{1}H-NMR spectra (Fig. 2) showed the formation of dihydropyrazole with its characteristic that has geminal and vicinal proton, i.e. H_a, H_b, and H_x. The protons were identified as doublet of doublet peaks in upfield. CH_2 geminal protons of pyrazoline ring commonly appeared as two doublet of doublet signals at 3.23-3.58 ppm (H_a) and 3.80-3.96 ppm (H_b) while the proton signal of CH (H_x) appeared at 5.57-5.74 ppm [18]. Signal 1 and 4 were identified as signals from CH_2 protons, i.e. H_a and H_b. Signal 5 corresponding to vicinal proton of CH (H_x) that coupled by two geminal protons attached to C4 pyrazoline ring. Signals 11 and 12 with each integration equivalent to 2 H predicted as aromatic ring$_1$ protons. Signal 12 that had chemical shift higher than 11 suspected as proton which was closest to the nitro group. Proton on the aromatic ring$_1$ appeared in the area downfield due to the effect of the nitro group that attached. Protons on the aromatic ring$_2$ appeared as a signals of 6 and 10. Signal 10 appeared in upfield due to nitro group at ortho position. Protons on aromatic ring$_3$ appeared as 7, 8 and 9 which have almost the same value of *J*. Signal 7 which splitted as triplet predicted as proton of aromatic ring$_3$ in C_3 which had two protons neighbor. Proton 7 and 8 that had integration of 2 protons were protons from aromatic ring$_3$. From ^{1}H-NMR spectra, it could be said that pyrazoline has been formed. Besides that, the loss of unsaturated α-β keto group that was indicated by there was no signal with 15 Hz of *J* value.

FIGURE 1. Product synthesis of pyrazoline **3**

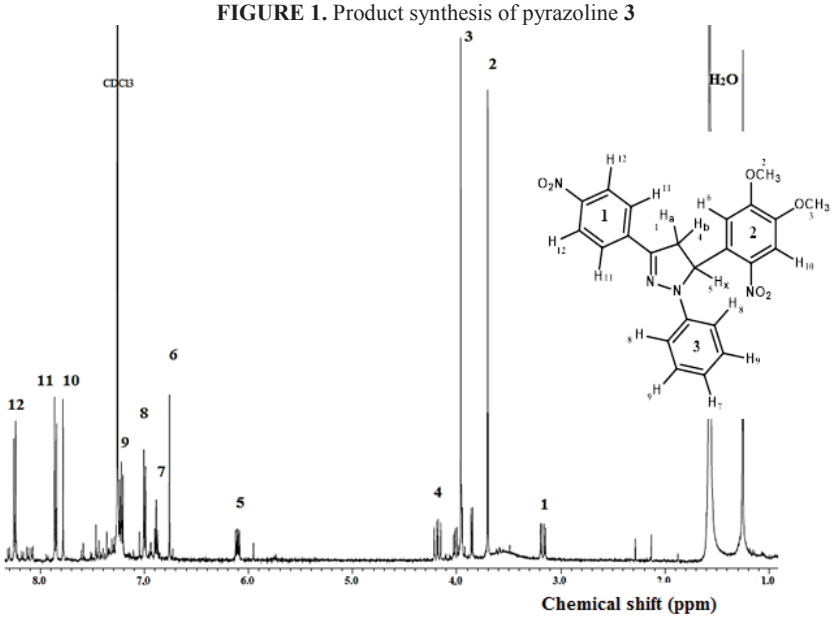


FIGURE 2. ^{1}H-NMR spectra of pyrazoline **3**

The interest in the IR spectra of the compound presented in Fig. 3, lies mainly in the bands due C=N and C-N functional groups. Band appeared at 1597 cm^{-1} was assigned to ν(C=N) because of ring closure[15]. A weak adsorption appearing at 1072 cm^{-1} was attributed to the C-N stretching vibrations, which also confirm the formation of desired pyrazoline ring. In addition, the formation of product reinforced by the loss of absorption band ν(C = C) of enone group. Comparison of IR spectra of the reactant and product were presented in Figure 3.Thesynthesis of pyrazoline from chalcone and hydrazine was predicted through the mechanism of 1,3-dipolar cycloaddition [16] based on the reaction mechanism, shown in Fig. 4.

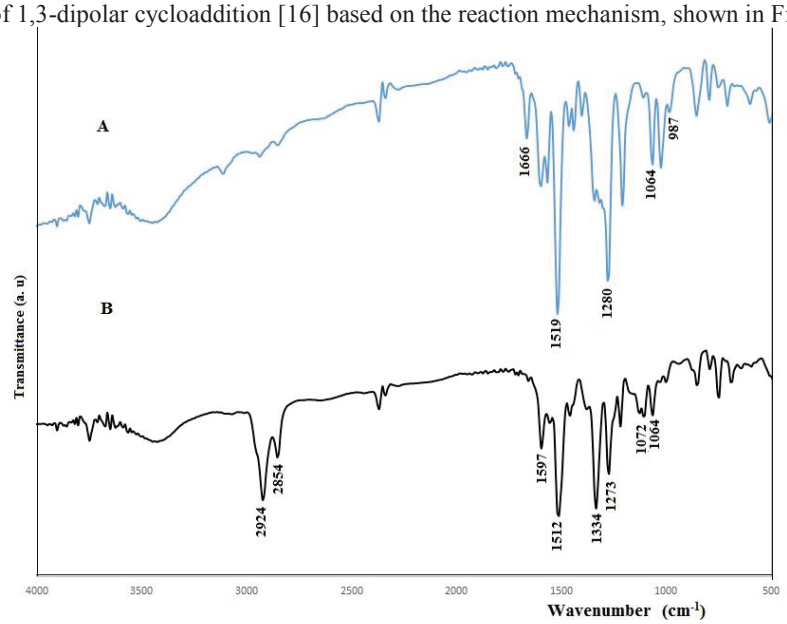


FIGURE 3. IR spectra of chalcone (A) and pyrazoline (B)

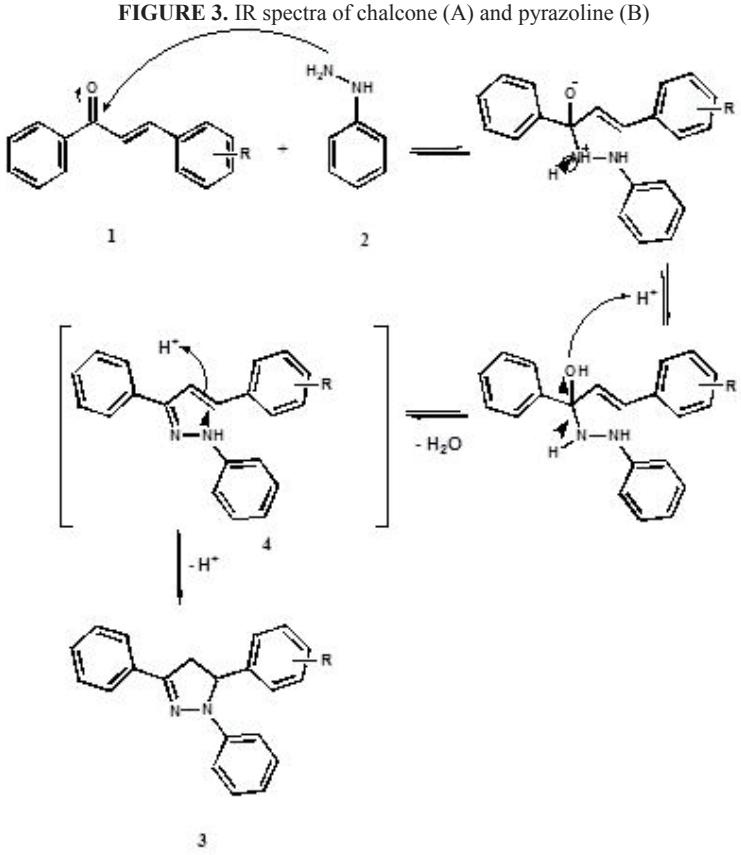


FIGURE 4. Reaction mechanism of pyrazoline **3** via cycloaddition reaction

The reaction mechanism began with nucleophilic addition at C=O of enone group chalcone **1**, then followed by dehydration, to form intermediate (hydrazone **4**). The formation of hydrazone was marked by the signal proton at 1.25 ppm corresponded to hydrazone **4** proton. Hydrazone **4** undergo cyclization with the attack of lone pair of N_1 on C_β enone group which was called rate determining step [19]. Electropositivy of C_β affected the rate of the cyclization reaction. Nitro group that attached at aromatic $ring_1$ was predicted reduce the density of electron at C=O of enone that could accelerate the occurrence of reaction. This was the reason that made this reaction did not need acetic acid. Moreover, when the reaction was tried in acid medium, the product did not form due to the prediction that there were decomposition or formation of nitroso group. It was proved by production of blackish solid product that has similar color when nitro group turned into nitroso.

Antibacterial Activity

Pyrazoline compound **3** did not showed antibacterial activity against *S. aureus*, *B. cereus* and *E. coli*. However, it had activity against *S. flexneri* with diameter of inhibition zone (DIZ) 9.25 mm at 1000 ppm (shown in Fig. 5 and Table 1). Against *B. subtilis*,it showed the greatest DIZ at 500 ppm, 5.75 mm. The antibacterial activity of compound can be affected by substituents. Pyrazoline did not exhibit good activity against *E. coli,* in line with research conducted by Baluja and Chanda[13] which reported that *E. coli* was difficult to be inhibited by pyrazoline. Chauhan et al.[20] said that the Gram-positive bacteria were more easily inhibited because their outer peptidoglycan layer which easily penetrated with less effective permeability whereas Gram-negative bacteria have an outer membrane phospholipid with lipopolysaccharide structure. However, the pyrazoline compound that had been synthesized had biggest DIZ against *S. flexnerri* which was well kwon as negative bacteria. It was observed that the pyrazoline has weak activity against all tested strains. That was predicted due to the substituents that attached (especially three aryls that closed to pyrazoline ring) and incompatibility with strain of bacteria, regardless of pyrazoline ring as lead compound. It was appropiate with the research that has been performed by Ozdemir et al.[21] which showed that pyrazoline derivatives had wide range of MIC, i.e. 3.9-250 mg/mL against all evaluated strains. The antibacterial activity can be explained by mechanism of action that was affected by the capability of active compounds in bacteria cell to form interaction with protein of target. The difference of substituens affected the activity because they were the side that enabled to interact with protein target of bacteria cell. The interactions that may be occurred were hydrogen bonding, ionic and liphopilic[10]. In addition, the strains also worked on the activity due to selectivity of active compounds.

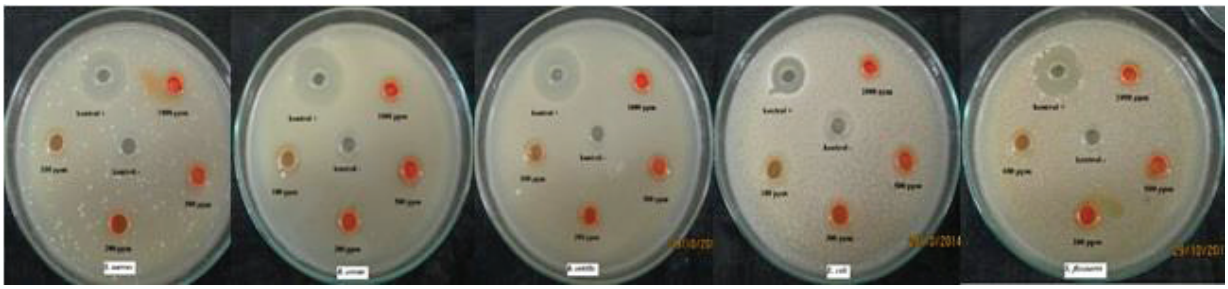
It was predicted that the nitro groups had role towards antibacterial activity. They could be interaction site of compound and protein from bacteria or the other reason that be related with lipophilicity of compound. The compound that has suitable lipophilicity can diffuse into cell and perform its role to combat the bacteria.

TABLE 1. Antibacterial activity of pyrazoline **3**

Concentration (ppm)	Diameter of Inhibition Zone (mm)				
	Staphylococcus aureus	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Shigella flexneri*
100	-	-	4.50	-	-
300	-	-	3.75	-	6.25
500	-	-	5.75	-	7.25
1000	-	-	3.75	-	9.25
Control (-)	-	-	-	-	-
Control (+)	13.50	17.25	20.75	8.50	12.25

Control (+): tetracycline 100 ppm, control (-): DMSO, (-): did not show antibacterial activity

FIGURE 5. Antibacterial test of pyrazoline **3**



CONCLUSION

Pyrazoline has been succesfully synthesized as red solid, in 33.06% yield via cycloaddition reaction. The compund has antibacterial activity against *B. subtilis* and *S. flexneri*. The nitro groups that substituted in aromatic ring were predicted as a part of pharmacophore. However, it should be investigated further.

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